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PROTEIN QUALITY IN HUMANS:

ASSESSMENT AND IN VITRO ESTIMATION

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Part III In Vitro Methods for Assessing Protein Nutritional Value

Collaborative Studies of Amino Acid Analyses: A Review and Preliminary Observations from a Nine-Laboratory Study

M.L. Happich, C.E. Bodwell and J.G. Phillips

The use of amino acid composition data has become increasingly important in the assessment of protein nutritional quality of food products, particularly of new food products. The contents of individual essential amino acids in foods are used in calculating Food and Agriculture Organization (FAO) amino acid scores and chemical scores and predicting protein efficiency ratios by use of mathematical equations (Alsmeyer *et al.*, 1974; Happich *et al.*, 1975; Lee *et al.*, 1978; Satterlee *et al.*, 1979). Essential amino acid composition data may be used as a basis to enrich foods with individual amino acids and to combine protein sources for complementation and mutual supplementation of the proteins, with an enhancement of nutritional value (Bressani, 1977). These and other uses of amino acid data will increase in the future. In particular, amino acid composition data are potentially important for use in assay methods for nutritional labeling and demonstrating compliance with FDA and USDA product standards (See Chapters 4, 5, and 6). The potential importance of amino acid determination in food analysis requires standardization of procedures for the preparation of protein food samples for amino acid analysis, and knowledge of the inter- and intralaboratory variation of procedures used to hydrolyze the protein in food sources and of the subsequent analysis of individual amino acids. These requirements are particularly important if amino acid analysis data are used to calculate the protein nutritional value of food sources for nutritional labeling, regulatory purposes, fortification of foods, or mutual supplementation of foods.

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In the United States there are no official methods for sample preparation prior to hydrolysis or for hydrolysis of the proteins prior to amino acid analysis. Each laboratory is free to use available methods and procedures of choice and to modify them as desired. The only official methods for determining amino acid composition in food in the United States are a titration method for free amino acids in lemon juice (AOAC, 1980; Sec. 22.106), one for proline in honey (AOAC, 1980; Sec. 31.116), and one for available lysine which includes hydrolysis of the protein (AOAC, 1980; Sec. 43.224).

STUDIES ON ION-EXCHANGE AMINO ACID ANALYSES

Collaborative studies on variation of the methods for determining amino acids have been reported by Porter *et al.*, 1968; Derse, 1969; Knipfel *et al.*, 1971; Cavins *et al.*, 1972; Westgarth and Williams, 1974; Williams *et al.*, 1979; and most recently Sarwar *et al.* (see Chapter 13).

Porter and colleagues in Great Britain conducted assays on a standard mixture of amino acids in solution and on two test proteins, gelatin and freeze-dried cod muscle. Their objective was to assess the precision of the ion-exchange column chromatographic technique, including the manual procedure, as operated in seven different laboratories. They concluded that the automatic amino acid analyzers afford better precision than the manual procedure, but that experienced workers could obtain broadly similar results with the manual procedure. The absolute mean deviations were lower for the standard mixture of amino acids than for the test proteins, gelatin and cod muscle. Seven of the nine collaborators recovered 81.5 to 88% of the total Kjeldahl nitrogen of the cod muscle as amino acid nitrogen. For gelatin, methionine and tyrosine had high mean absolute deviations ($>\pm 10\%$).

Derse (1969) reported a study in which a sample of 50% protein soybean meal was issued to 12 collaborating laboratories proficient in the use of amino acid analyzer techniques. The results of the test, which were considered good, illustrated differences in percent of each amino acid found in the 50% protein soybean meal. Methionine and cystine showed the greatest differences between laboratories. Methionine ranged from a trace to 0.72% and cystine ranged from 0.44% to 1.36%. Standard deviations and coefficients of variation were not calculated.

Knipfel *et al.* (1971) studied the analysis of 2 hydrolyzates of each of 3 proteins (casein, soy flour, and fish flour) prepared by Knipfel and sent to five cooperating laboratories. Duplicate analyses of individual hydrolysates within laboratories produced coefficients of variation of 3.7 to 4.4% (Knipfel *et al.*, 1971). There was a significant hydrolyzate-amino acid interaction for soy flour and casein. The concentration of some amino acids was higher in one hydrolyzate and the concentration of others was higher in the second hydrolyzate. The methionine content of casein showed a difference of about 30% between the two hydrolyzates. Practically identical results could be produced from a standard sample tested in the different laboratories by varying analytical procedures. Adjustment of the mean values for amino

acid concentrations for each protein source within individual laboratories to constant total recovery of 90g/16g N reduced the interlaboratory variation considerably.

Kwolek and Cavins (1971) examined variability in published amino acid data on plant seed or seed products from 18 selected references. Cysteine, tryptophan, tyrosine, and methionine had higher relative standard deviations than the other 14 amino acids. A mean relative standard deviation of about 8% (ranging from 5.9 to 14.1, depending on the amino acid) was observed between samples.

Cavins *et al.* (1972) conducted an interlaboratory study with five laboratories on the amino acid analysis of soybean meal. The analysts evaluated four methods of hydrolysis: in sealed ampoules, in sealed flasks, and with two acid-to-sample ratios by the reflux method. Later, they evaluated the effect of mesh size (40-, 80-, over 70-, and under 270-mesh) of the soybean meal on the amino acid analysis results. Interlaboratory variations were significant for twelve amino acids and ammonia in the hydrolysis study and for all (18) amino acids and ammonia in the study on mesh size. Tryptophan results obtained by four procedures on 80-mesh samples were in good agreement. Analysis of the 80-mesh samples for cystine by two procedures, one an oxidation and the other a reduction followed by a derivatization, produced values that did not agree. Normalization of results to 95% nitrogen recovery had only a small effect on the statistical analysis.

Westgarth and Williams (1974) compared the methods of Miller (1967) and of Spies and Chambers (1949) for the determination of tryptophan on groundnut, soya bean, and cottonseed meals at eight laboratories. Tryptophan values obtained by both methods were similar. The collaborators preferred the Miller method because it appeared to provide a satisfactory estimation of tryptophan in feedstuffs.

Williams *et al.* (1979) reported determinations of "cyst(e)ine" in feedstuffs in eight laboratories by the Moore (1963) performic acid oxidation (PAO) method and the Spencer and Wold (1969) dimethyl sulfoxide (DMSO) method. Casein, fishmeal, extracted leaf protein concentrate, groundnut, soya bean, and wheat were the protein sources analyzed. The authors state that the "variation between duplicate hydrolyses within laboratories was similar by both methods (mean coefficients of variation 4.3 and 5.0%) and smaller than the variation between laboratories (mean coefficients of variation 14.8 and 14.3%)." Apparently both methods were considered to be satisfactory for estimating "cyst(e)ine" in the samples tested except for casein. Six of the eight laboratories found significantly more "cyst(e)ine" in casein by the DMSO method. Although the DMSO method was faster, the PAO method was preferred by the collaborators because methionine could be estimated at the same time.

Past collaborative studies were important at the time they were conducted and are historically important now. However, they were usually either limited in scope, did not include hydrolysis procedures for all amino acids, did not rigorously standardize procedures used or did not analyze a variety of protein sources.

The collaborative study on amino acid analysis recently completed in Canada by Sarwar *et al.* is described in Chapter 13.

METHODOLOGY CONSIDERATIONS

The preparation of the sample, hydrolysis methods, hydrolysis time, resolution of some amino acids and/or amino acid derivatives, and a reference protein for amino acid analysis required consideration when a protocol was developed for the current amino acid analysis collaborative studies. Methodology selected for the current collaborative studies is given below.

Sample Preparation

Chemical analyses and particularly amino acid analyses of protein food sources must be made on homogeneous material. Meats, meat products, and variety meats which have a high content of water and fat should be lyophilized, defatted (the defatting solvent thoroughly removed), ground finely, mixed thoroughly, and sampled for proximate analysis and amino acid analyses at the same time. Petroleum ether is the solvent of choice to defat samples prior to amino acid analysis in the ERRC (Eastern Regional Research Laboratory) Meat Laboratory. We compared petroleum ether with acetone and diethyl ether for defatting of beef. We found no appreciable differences in amino acid analyses after the samples were defatted with any one of these three solvents (unpublished data). Removal of carbohydrate from protein food sources prior to amino acid analysis, although recommended by Block and Weiss (1956a), is not widely practiced today.

Hydrolysis of Proteins

The use of different procedures or conditions for the hydrolysis of proteins by different investigators is a well-known source of variability in the analyses (Block and Weiss, 1956b; Blackburn, 1978). Hydrolysis with 6 N HCl is a widely used method of choice except for the determination of tryptophan. Although some analysts determine cystine and methionine in the usual routine acid hydrolyzates, it is not the preferred method. Great care must be taken to prevent oxidation during hydrolysis with hydrochloric acid, removal of hydrochloric acid after hydrolysis and evaporation of the hydrolyzate to dryness. Hackler (1974) suggested that greatest care should be taken in the removal of air from the hydrolysis sample and tube. Even so, cysteine is subject to oxidation and cystine is racemized to meso-cystine and DL-cystine during acid hydrolysis and chromatography (Friedman and Noma, 1975). Cysteine and cystine react with tryptophan, carbohydrates and other substances commonly present during hydrolysis (Friedman and Noma, 1975). Methionine and cystine contents of a protein sample can be determined fairly accurately by oxidation with performic acid, followed by hydrolysis with 6 N HCl, and analyzed by an ion-exchange procedure (Walker *et al.*, 1975; Shram *et al.*, 1954; Neumann *et al.*, 1962; Moore, 1963). This method is preferred for the determination of methionine and cystine.

Tryptophan is extensively destroyed during hydrochloric acid hydrolysis of proteins, producing ammonia as the only recognizable end product (Friedman and Finley, 1971; Friedman and Finley, 1975). Basic hydrolysis of proteins is used by most analysts for the determination of tryptophan. By the method of Hugli and Moore (1972) (hydrolysis with 4.2 N sodium hydroxide), partially hydrolyzed potato starch is added as an antioxidant to protect the tryptophan (Dreze, 1960; Hugli and Moore, 1972). Oelshlegel *et al.* (1970) suggested the use of a polypropylene or polyallomer tube, resistant to hot alkali, inside a glass tube for the hydrolysis.

Hydrolysis Time

Hydrolysis time, another potential variable, has been selected by various analysts rather arbitrarily. For convenience, the times most often used have been 18, 21, 22, or 24 hr (with 6 N HCl). Cherry (1978; 1979) found that variations in the release of amino acids from cottonseed protein products are a function of hydrolysis time. When samples were hydrolyzed with 6 N HCl for 8, 16, 24, 32, 48, and 72 hr, the release of amino acids increased between 8 and 16 hr and decreased between 16 and 24 hr, leveled off between 24 and 32 hr, and then increased to optimum levels at 48 hr. Little change or a decline in amino acid content was observed between 48 and 72 hr. The amount of change varied greatly among amino acids.

Hackler (1971) studied the release of methionine from pea beans, beef round, and gelatin by hydrolysis with 6 N HCl for 5½, 11, 22, 44, or 66 hr. Twenty-two hr of hydrolysis produced the highest value for methionine from pea beans; there were only small differences in the methionine released from beef round in 11, 22, 44, or 66 hr; the methionine values obtained for gelatin were highest (and similar) after either 44 or 66 hr of hydrolysis.

Hackler *et al.* (unpublished data) also studied the release of amino acids from the proteins of rice (Table 12.1) by hydrolysis with 6 N HCl for 6, 12, 24,

TABLE 12.1. SPECIFIC AMINO ACID CONTENT (GRAMS AMINO ACID/16G NITROGEN) OF TWO PROTEIN SOURCES AS AFFECTED BY VARYING HYDROLYSIS TIME

	Lyophilized defatted beef[1]				Rice[2]				
	Hydrolysis time, hr				Hydrolysis time, hr				
Amino acid	18	21	24	48	6	12	24	48	72
Histidine	3.8	3.8	3.8	3.9	2.1	2.2	2.1	2.3	2.2
Isoleucine	4.8	4.9	4.7	4.9	3.1	3.4	4.0	4.5	4.6
Leucine	8.4	8.2	8.2	8.3	7.7	8.2	8.1	9.0	8.7
Lysine	8.9	8.9	9.1	8.8	3.0	3.3	3.3	3.5	3.4
Methionine	3.5	3.4	3.0	2.6	2.3	2.4	2.4	2.4	2.4
Phenylalanine	4.4	4.5	4.4	4.4	4.4	4.7	5.1	5.3	5.1
Threonine	4.6	4.3	4.5	4.3	3.0	3.0	3.4	3.1	2.9
Tyrosine[3]	3.8	3.7	3.8	3.9	2.6	2.9	3.1	3.0	3.0
Valine	5.0	5.0	5.0	5.2	4.8	5.4	6.1	6.6	6.4

[1]M.L. Happich, unpublished data; acid hydrolyzate.
[2]L.R. Hackler et al., unpublished data; acid hydrolyzate.
[3]Tyrosine is not an essential amino acid but may replace part of the phenylalanine requirement for humans (Rose and Wixon, 1955).

48 and 72 hr. Valine and isoleucine showed the greatest overall differences in recovery with variation in hydrolysis time. The highest values were recovered by 48 or 72 hr, respectively, and were 8 and 15% higher, respectively, than values obtained after a 24 hr hydrolysis. Methionine recovery varied least reaching the highest value by 12 hours hydrolysis and remaining constant thereafter. Threonine and tyrosine recoveries were best from a 24 hr hydrolysis. Forty-eight hr hydrolysis showed the highest values for histidine, leucine, lysine and phenylalanine, i.e., 9.5, 11.1, 6.0 and 3.9% higher, respectively, than the values recovered by 24 hr hydrolysis.

Although not part of the collaborative study, a time study is underway at ERRC, USDA, to evaluate the release of amino acids from the proteins of the food sources in the collaborative study after 18, 21, 24, and 48 hr of hydrolysis. Results for lyophilized, defatted beef (Table 12.1), indicate that, in general, the largest differences occurred in the 48 hr samples. Methionine and threonine show a decrease of 25.7 and 6.5%, respectively, after 48 hr hydrolysis as compared with values after 18 hr. Valine shows an increase of 4.0%. Changes in the other amino acids varied from 1.1 to 2.6%, values within experimental error.

Reference Protein for Amino Acid Analysis

Most analysts use an amino acid reference standard. Commonly, a reference protein is used to determine the accuracy of the recovery of amino acids from the protein and of the analytical methods used. The selection of a reference protein for amino acid analysis of food sources is difficult because the protein content of a food source is usually a mixture of individual proteins. These individual proteins are seldom available in purified form and are often of unknown proportions. Because other constituents such as carbohydrates, mineral salts, other naturally occurring materials, and materials added during manufacture may produce reactions with, or changes in, the hydrolyzed amino acids, a good general standard is lacking for protein food sources.

CURRENT COLLABORATIVE STUDY

Nine laboratories participated in the current collaborative study: Campbell Soup Company; General Mills, Inc.; Kraft, Inc.; Mead Johnson; Northern Regional Research Center, (USDA); Procter and Gamble Company; Quaker Oats Company; Ralston Purina Company; and the University of Nebraska. In a tenth laboratory, Nabisco, Inc., amino acid compositions were determined on the same pretest and test samples by gas chromatography for comparison with results from the automated ion-exchange chromatographic analyzers.

Objectives and Design

The objectives of the study were:

1. Standardize the preparation of protein food sources for amino acid analysis, including the hydrolysis of proteins.
2. Compare two different approaches for normalizing amino acid analysis data.
3. Determine the inter- and intralaboratory variation for the analysis of individual amino acids in selected protein food sources.

The experimental protocol is set forth in Table 12.2.

Sample Preparation and Storage

The samples for the current collaborative study were purchased or prepared as indicated: The pretest samples, tuna fish and peanut flour, were originally obtained for use in studies by Bodwell *et al.* (1978); textured soy protein was obtained in the spring of 1979 and ground; casein was a sample of the ANRC (Animal Nutrition Research Council) casein purchased in January, 1979. These four samples were stored at −20°C until shipped to ERRC (USDA) in August, 1979. The non fat dried milk powder (low heat, spray dried, containing no added nutrients); the wheat flour (commercial bakers' bread flour of hard, red spring wheat, enriched with niacin, iron, thiamine, and riboflavin); and the sample of lean beef (semitendinosus muscle) were all obtained in September–November, 1979. The beef was lyophilized, defatted with petroleum ether, and ground in a Wiley Mill to pass a 2-mm screen. All samples were thoroughly mixed several times; fifteen 30g samples were placed in air tight bottles, sealed for use in the amino acid analysis, and stored just above freezing at about 4°C or less. One sample was taken for proximate analysis on each protein source. Nitrogen was determined by the macro Kjeldahl method in quintuplicate (AOAC, 1980; Sec. 24.027; Sec. 2.057).

Hydrolysis of Proteins

The hydrolysis procedures for the current study were specified in much detail to minimize interlaboratory variation. Either the stopcock style glass flask assembly or a drawn glass tube was allowed as the container for the hydrolysis. The contents (protein source + 6 N HCl) were frozen by placing the lower portion of the flask or tube in a dry ice-acetone bath. The flask or tube was evacuated of air by a high vacuum pump, the vacuum was then closed off and the contents were thawed. These steps were repeated twice. When the flask assembly was used, the stopcock also was evacuated of air. Finally, the flask was closed by the stopcock or the tube was sealed by use of a Bunsen burner. Based on the work of Hackler (1971) and others, 22 hr hydrolysis time at an oven temperature of 110°C was selected. For trypto-

phan analyses, the samples were hydrolyzed with 4.2 N NaOH by the method of Hugli and Moore (1972) in a stopcock style test-tube assembly or a drawn test tube. A polypropylene tube was inserted inside the tube to hold the protein sample and other hydrolysate components. Otherwise the samples were treated the same as for the hydrochloric acid hydrolyses for evacuation of air, hydrolysis time and temperature (Table 12.2). Collaborators were requested to run duplicate hydrolyses on each protein source.

TABLE 12.2. EXPERIMENTAL PROTOCOL FOR AMINO ACID ANALYSIS COLLABORATIVE STUDY

Protein Sources

Pretest Samples: Tuna
Peanut flour

Test Samples: ANRC casein
Freeze-dried defatted beef (semitendinosus muscle)
Non fat dried milk
Wheat flour (commercial bakers' bread flour)
Textured soy protein

Hydrolysis (in duplicate; under vacuum):

6 N HCl[1]
30–40 mg protein (N × 6.25)
10 ml 6 N HCl

Performic acid pretreatment (Moore, 1963) followed by 6 N HCl[1]

4.2 N NaOH (Hugli and Moore, 1972)
8–10 mg protein (N × 6.25)
1.0 ml 5 N NaOH
1.0 ml isopropyl alcohol
Partially hydrolyzed potato starch (25 mg; for casein, beef, milk, and tuna only)

17 Common amino acids,
Hydroxylysine,
Hydroxyproline,

Methionine as methionine sulfone
Cysteine as cysteic acid

Tryptophan

Time: 22 hr

Temperature: 110°C

Analyzer: Ion-exchange chromatography—9 collaborators (each by use of own system)
Gas chromatography—special collaborator

Buffers: Na citrate
or
Li citrate

Amino acid hydrolyzates analysis: analyzed in duplicate

Calibration standards:
For 17 common amino acids and ammonia (collaborator's own standard and a common standard)

For cysteic acid and methionine sulfone (collaborator's own standard and a common standard)

Reporting of data: as grams amino acid/16g N (each collaborator's standard use as a reference)

[1]Hydrolyzed by same conditions.

Amino Acid Analysis

Each collaborator used the analyzer and buffer system normally used in his/her laboratory, a recognized variable we could not change. We requested collaborators to run duplicate amino acid analyses on each hydrolyzate so that an estimate could be made of the within instrument variability of each analyzer. The data were calculated as grams amino acid per 16g of nitrogen and returned to us. The choice of 16g nitrogen is not meant to imply that this is equivalent to 100g of protein in the case of each protein source.

Resolution of Methionine Sulfone

We found that the current collaborators had a preference for using lithium acetate rather than sodium acetate buffers to resolve methionine sulfone in the performic acid oxidized 6 N HCl hydrolyzates. The difficulty with the sodium system lies in its incomplete resolution of methionine sulfone, aspartic acid, and threonine. The chromatogram obtained by a 24-hr separation procedure, Figure 12.1, was taken from Moore *et al.* (1958). The elution times for aspartic acid, methionine sulfone, and threonine were well separated with methionine sulfoxide eluted prior to aspartic acid. However, in a 90-min analysis procedure by Greenberg at ERRC

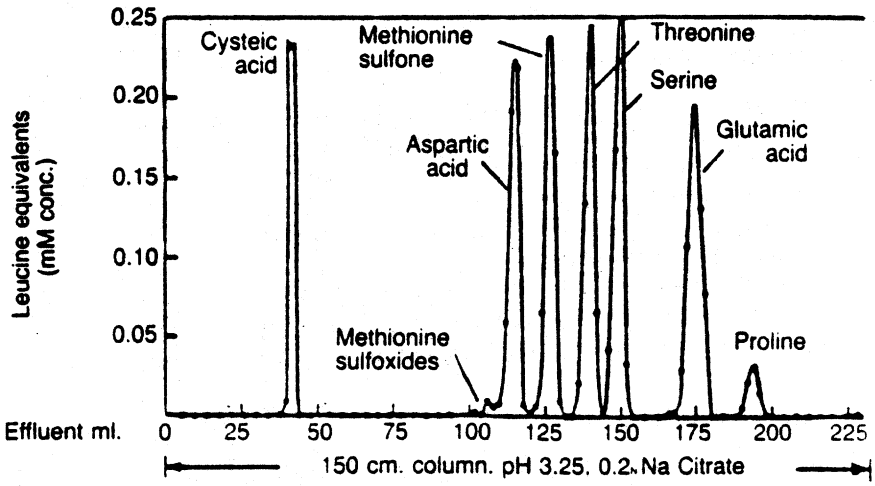

FIG. 12.1. CHROMATOGRAPHIC FRACTIONATION OF MIXTURE OF STANDARD AMINO ACIDS ON COLUMN OF AMBERLITE IR-120. REPRINTED WITH PERMISSION FROM: ANALYTICAL CHEMISTRY 30(7): 1185, 1958. MOORE, S., SPACKMAN, D.H., AND STEIN, W.H. CHROMATOGRAPHY OF AMINO ACIDS ON SULFONATED POLY STYRENE RESINS. COPYRIGHT BY THE AMERICAN CHEMICAL SOCIETY

(Figure 12.2), aspartic acid and threonine eluted only 2.5 min apart, making it difficult to place methionine sulfone well resolved between the two. Talley, Plant Products Laboratory, ERRC, regularly and successfully analyzes for methionine sulfone and methionine sulfoxide using a sodium citrate buffer at pH 3.25 in a 3¾-hr procedure. The sulfoxide is eluted about 2 min before the aspartic acid. He keeps his conditions precisely the same from sample to sample to obtain good separations consistently.

At the ERRC Meat Laboratory, we regularly use a sodium citrate buffer at pH 2.9 to elute hydroxyproline at 38–39 min, which is about 6 min before aspartic acid emerges. The column is 0.9 cm in diameter and contains 31.5 cm of a cation exchange resin. With this buffer, methionine sulfone coelutes with aspartic acid. Although it is possible to elute methionine sulfone from the column before aspartic acid, it must precede methionine sulfoxide and not interfere with hydroxyproline. Each laboratory had to work out this separation problem. A lithium acetate buffer system was recommended if a laboratory had an extra column available. The run could be aborted as soon as the amino acids required were eluted.

Normalization of Data

ANRC casein, one of the proteins in this test, was selected as the standard protein for this study. Upon completion of the study, amino acid analysis data will be normalized against a "best" analysis of casein and against total Kjeldahl nitrogen recovery. Determined ammonia will be included in the calculation of total nitrogen recovery. Inter- and intralaboratory variation will be determined before and after casein or nitrogen normalization.

Calibration Standards

As suggested by Yates, Campbell Soup Company, a calibration standard for the 17 common amino acids and ammonia and a calibration standard for cysteic acid and methionine sulfone were sent to all collaborators. The analysis of a common standard will allow for interpretation of the data upon completion of the study. Each standard was to be analyzed against the appropriate calibration standard solution each collaborator used for analysis of the test samples. These data will allow us to determine whether the standard was a source of variation in the analysis and will allow for possible adjustment of the data obtained by a common standard.

Statistical Analyses

Standard deviations and coefficients of variation were determined for the lysine and valine data obtained from the two pretest samples (tuna fish and peanut flour) and from the five test samples (ANRC casein, textured soy protein, wheat flour, defatted lyophilized beef, and non fat dried milk powder), with and without identified outliers. Outliers were identified by

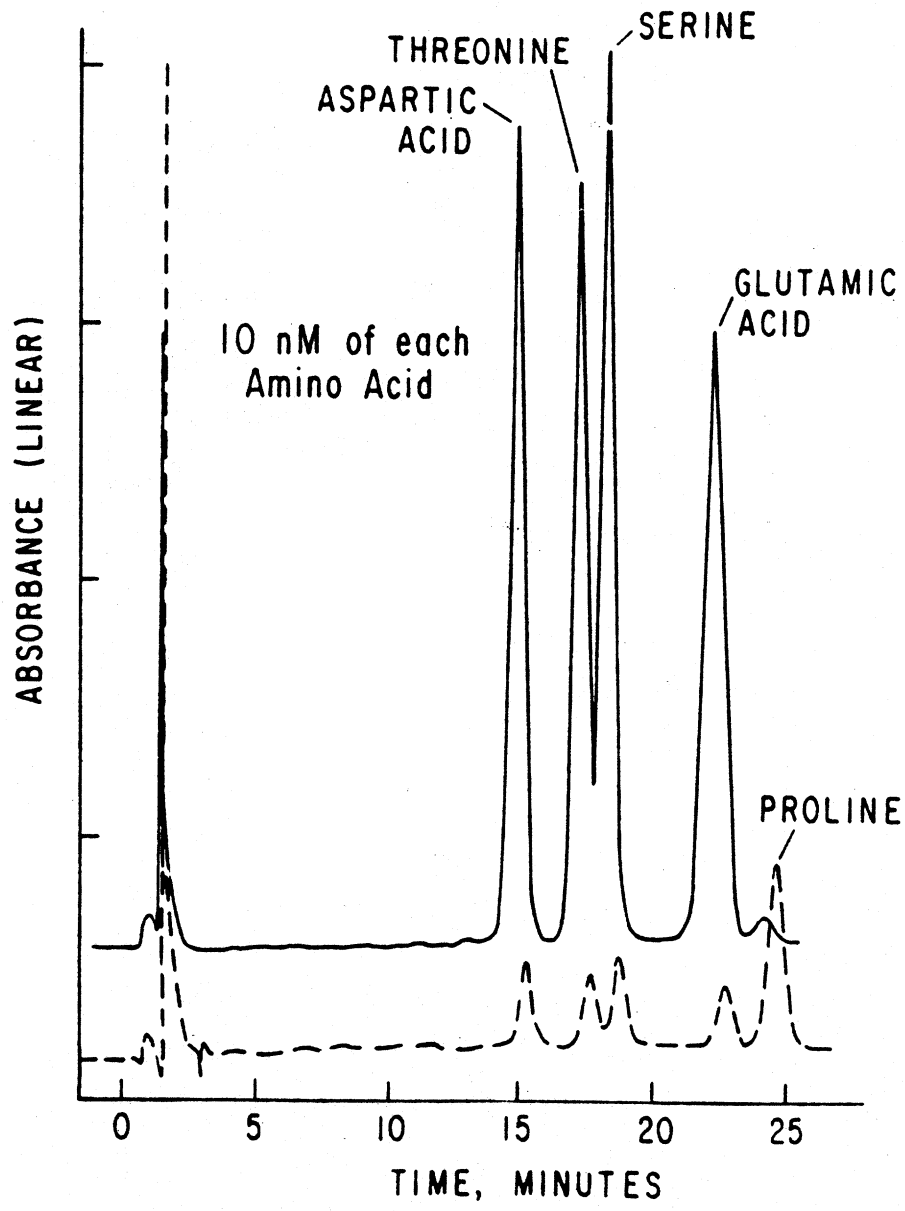

FIG. 12.2. PARTIAL CHROMATOGRAM OF AMINO ACID CALIBRATION STANDARD FROM 90-MIN AMINO ACID ANALYSIS. (R., GREENBERG, EASTERN REGIONAL RESEARCH CENTER, USDA).

the method of Anscombe and Tukey (1963). An analysis of variance was done on the data for all amino acids from the two pretest samples and for lysine from the five test samples.

Results from the Pretest Samples

The collaborators are enthusiastic and are making an effort to return the data promptly. During this study we have encountered the usual problems of amino acid analysis. Several of the collaborators who do not commonly use the performic acid-6 N HCl procedure for methionine and the 4.2 N NaOH hydrolysis procedure for tryptophan spent time becoming familiar with the method, the primary reason for sending out two pretest samples. The return of the data from the pretest samples took longer than anticipated. To date, we have received pretest data from eight laboratories and the special collaborator. Only three laboratories have submitted results for methionine sulfone and three for tryptophan. Six laboratories reported the analysis of cystine as cysteic acid (Table 12.3). At least two of those who had analyzed for methionine sulfone used lithium acetate buffers and the third used sodium acetate buffers.

TABLE 12.3. METHIONINE, CYSTINE, AND TRYPTOPHAN DATA FOR PRETEST SAMPLES[1]

	Grams amino acid/16g nitrogen						
	Laboratories						
Amino acid	2	3	4	5	6	8	S.D.
	Tuna						
Methionine	2.40	—	2.84	—	—	—	—
½ Cystine	0.91	1.07	0.92	0.69	0.49	1.05	0.22
Tryptophan	—	—	1.35	1.07	0.89	—	0.23
	Peanut flour						
Methionine	1.15	—	1.32	—	—	1.09	0.12
½ Cystine	1.14	1.64	1.28	1.01	1.74	1.58	0.30
Tryptophan	—	—	0.58	0.72	0.48	—	0.12

[1]The data have not been normalized (see text).

In the determination of lysine in the two pretest samples, tuna fish and peanut flour (Table 12.4), one laboratory (Laboratory 5) had very high values for tuna fish and another (Laboratory 3) had low values for peanut flour. Statistical data (Table 12.5) show that the interlaboratory standard deviations (S.D.) for reproducibility of the lysine determinations were 0.95 from tuna and 0.37 from peanut flour. The coefficient of variation (C.V.) for each source is similar: 11.3% for tuna and 11.1% for peanut flour. Interlaboratory precision (reproducibility) for the determination of lysine in the pretest is lower than desirable. However, when the high lysine value for tuna (Laboratory 5) is treated as an outlier, the C.V. is lowered to 6.3% (Table 12.5). Likewise, removing the low value for lysine in peanut flour (Laboratory 3) lowers the C.V. to 7.6%. Both the very high and the very low values for lysine are outliers by the method of Anscombe and Tukey (1963).

TABLE 12.4. LYSINE DATA FOR PRETEST SAMPLES[1]

	Hydrolyzate[2]			
	Grams amino acid/16g nitrogen			
	Tuna		Peanut flour	
Laboratory	1	2	1	2
1	8.52	8.55	3.18	3.21
2	7.43	7.79	3.74	3.61
3	8.25	8.87	2.56	2.66
4	8.55	8.35	3.20	3.15
5	10.48[3]	10.43[3]	3.87[3]	3.78[3]
6	7.39	7.15	3.49	3.32
7	7.85	7.78	3.41	3.59
8	8.2	8.1	3.15	3.1

[1]The data have not been normalized (see text).
[2]Each value is the average of two analyses with the exception of those from Laboratory 5.
[3]Single analyses.

The intralaboratory precision (repeatability) of lysine determinations for tuna fish and peanut flour is high as indicated by the values shown in Table 12.5.

The interlaboratory precision of the valine determination for tuna fish and peanut flour is affected by the very low values obtained by one laboratory (Table 12.6). The C.V.'s for these protein sources were 20.8% and 19.0%, respectively (Table 12.7). Treating the very low results from Laboratory 6 as outliers (outliers by the method of Anscombe and Tukey, 1963) lowers the C.V.'s to 13.5 and 14.3%, respectively. The intralaboratory precision (S.D.) for the valine determinations is high, however, the relative precision (C.V.) is lower than that for lysine (Tables 12.5 and 12.7).

The analysis of variance for the pretest samples (two protein sources, duplicate 6 N HCl hydrolyses, and duplicate analyses of each hydrolyzate, no outliers) indicates that overall interlaboratory (reproducibility) S.D.'s for lysine and for valine (Table 12.8) were different from those of either tuna fish or peanut flour individually (Tables 12.5 and 12.7). In general the interlaboratory variation for all amino acids was high. Intralaboratory

TABLE 12.5. INTER- AND INTRALABORATORY VARIATION FOR LYSINE DETERMINATION OF PRETEST SAMPLES[1]

	Tuna		Peanut flour	
	8 Laboratories	Outlier removed	8 Laboratories	Outlier removed
Range, g AA[2]/16g N	7.15–10.48	7.15–8.87	2.56–3.87	3.1–3.87
Mean, g AA/16g N	8.36	8.05	3.31	3.44
S.D., g AA/16g N, (inter-)	0.95	0.51	0.37	0.26
C.V., % (inter-)	11.3	6.3	11.1	7.6
S.D., g AA/16g N, (intra-)	0.21	0.22	0.08	0.08
C.V., % (intra-)	2.5	2.8	2.4	2.3

[1]The data have not been normalized (see text).
[2]AA = amino acid.

TABLE 12.6. VALINE DATA FOR PRETEST SAMPLES[1]

Laboratory	Hydrolyzate[2]			
	Grams amino acid/16g nitrogen			
	Tuna		Peanut flour	
	1	2	1	2
1	5.18[3]	5.14	3.49[3]	3.54
2	4.52	4.53	3.41	3.31
3	3.45	3.92	2.53	2.47
4	5.15	5.15	3.35	3.10
5	5.56[3]	6.06[3]	3.58	4.66
6	2.84	2.60	2.21	2.19
7	4.79	4.87	4.12	4.02
8	5.35	5.30	3.75	3.55

[1]The data have not been normalized (see text).
[2]Each value the average of two analyses.
[3]Single analyses.

TABLE 12.7. INTER- AND INTRALABORATORY VARIATION FOR VALINE DETERMINATIONS OF PRETEST SAMPLES[1]

	Tuna		Peanut flour	
	8 Laboratories	Outlier removed	8 Laboratories	Outlier removed
Range, g AA[2]/16g N	2.60–6.06	3.45–6.06	2.19–4.12	2.47–4.12
Mean, g AA/16g N	4.65	4.93	3.29	3.44
S.D., g AA/16g N, (inter-)	0.97	0.66	0.63	0.49
C.V., % (inter-)	20.8	13.5	19.0	14.3
S.D., g AA/16g N (intra-)	0.18	0.17	0.15	0.16
C.V., % (intra-)	3.9	3.4	4.6	4.6

[1]The data have not been normalized (see text).
[2]AA = amino acid.

variation (repeatability) was also determined for each amino acid during the overall analysis of variance (Table 12.8). The C.V.'s for arginine, histidine, proline, and isoleucine ranged from 4.3 to 11.9%. Cystine had an extremely high C.V. of 31.6%. The C.V.'s of other amino acids were 3.8% or below. The analysis of variance showed that the laboratory-hydrolysis interaction was significant ($p < 0.05$) for several amino acids (i.e., aspartic acid, leucine, tyrosine, lysine, glutamic acid, alanine, and valine). However, only one, glutamic acid, showed a serious difference (~ 7%) between duplicate hydrolyses at a particular laboratory, while the other amino acids showed significant laboratory-by-hydrolysis interactions because of a difference in levels between laboratories. Laboratory-by-protein source interactions were significant ($p < 0.01$) for all amino acids except proline and were not significant for ammonia. These are due largely to the different amounts of amino acids found in the two samples and the variance of differences between laboratories.

TABLE 12.8. STANDARD DEVIATIONS (S.D.) AND COEFFICIENTS OF VARIATION (C.V.) FOR EACH AMINO ACID FROM DATA FOR TWO PRETEST SAMPLES COMBINED[1]

Amino acid	6 N HCl hydrolysis			
	Interlaboratory		Intralaboratory	
	S.D. g AA[2]/16g N	C.V.[3] %	S.D. g AA[2]/16g N	C.V.[3] %
Aspartic acid	1.19	12.4	0.24	2.5
Threonine	0.50	15.1	0.10	3.1
Serine	0.33	8.2	0.13	3.3
Glycine	0.50	10.4	0.18	3.6
Proline	0.44[4]	12.8	0.37[4]	10.7
Glutamic	1.26	8.6	0.26	1.7
Alanine	0.47	10.6	0.12	2.7
Valine	0.69	17.6	0.11	2.7
Isoleucine	0.79	22.8	0.41	11.9
Leucine	0.62	9.8	0.12	2.0
Tyrosine	0.38	10.8	0.14	3.8
Phenylalanine	0.35	8.2	0.15	3.4
Lysine	0.88	15.4	0.14	2.5
Histidine	0.53	6.6	0.29	8.4
Arginine	0.85	10.5	0.35	4.3
Ammonia	0.62[5]	52.3	0.47[5]	39.1
Cystine	0.46[5]	77.5	0.19[5]	31.6
Methionine	0.31[6]	15.3	0.07[6]	3.7

[1]From analysis of variance for 8 laboratories unless noted; no values (outliers) were excluded.
[2]AA = amino acid.
[3]C.V. (coefficient of variation) is standard deviation ÷ the mean value for each amino acid.
[4]Analysis of variance for six laboratories.
[5]Analysis of variance for five laboratories.
[6]Analysis of variance for four laboratories.

Preliminary Results from Five Test Samples

To date we have received amino acid analysis data from four collaborators on the five test samples (ANRC casein, textured soy protein, wheat flour, freeze-dried defatted beef, and non fat dried milk powder) hydrolyzed with 6 N HCl. Table 12.9 presents the statistical data for lysine. The highest interlaboratory variability (reproducibility) was observed for casein and defatted beef, principally due to the low lysine values obtained from one laboratory (Laboratory 4) for both protein sources (Fig. 12.3). The variability for the other three protein sources is under 5%. The statistical analyses were done on the data without any adjustments or normalization (nitrogen recovered or standard protein).

Intralaboratory variability (repeatability) shows moderate to high precision for each protein source (Table 12.9). The coefficient of variation of these five samples varied from 1.8 to 4.9%. All are acceptable values for high precision except the 4.9% value for the textured soy protein.

An analysis of variance was performed to determine the effect of laboratory, protein source, and hydrolysis, and their interactions on the determination of lysine. Significant interactions were found between laboratory and protein source and between protein source and hydrolysis. Laboratory-by-protein source interactions were apparent (Figure 3). The significant

TABLE 12.9. INTER- AND INTRALABORATORY VARIATION FOR LYSINE DETERMINATIONS OF FIVE TEST PROTEIN SOURCES[1,2]

Summary	Casein	Textured soy protein	Wheat flour	Defatted beef	Non fat d ied milk powder
Range, g AA[3]/16g N	6.35–8.40	5.34–6.18	1.62–1.91	6.95–8.53	6.81–7.84
Mean, g AA/16g N	7.75	5.88	1.82	7.78	7.38
S.D. g AA/16g N (inter-)	0.65	0.25	0.09	0.55	0.36
C.V., % (inter-)	8.4	4.2	4.9	7.0	4.9
S.D. g AA/16g N (intra-)	0.23	0.29	0.07	0.27	0.13
C.V., % (intra-)	3.0	4.9	3.8	3.5	1.8

[1]The data have not been normalized (see text).
[2]Data from four laboratories.
[3]AA = amino acid.

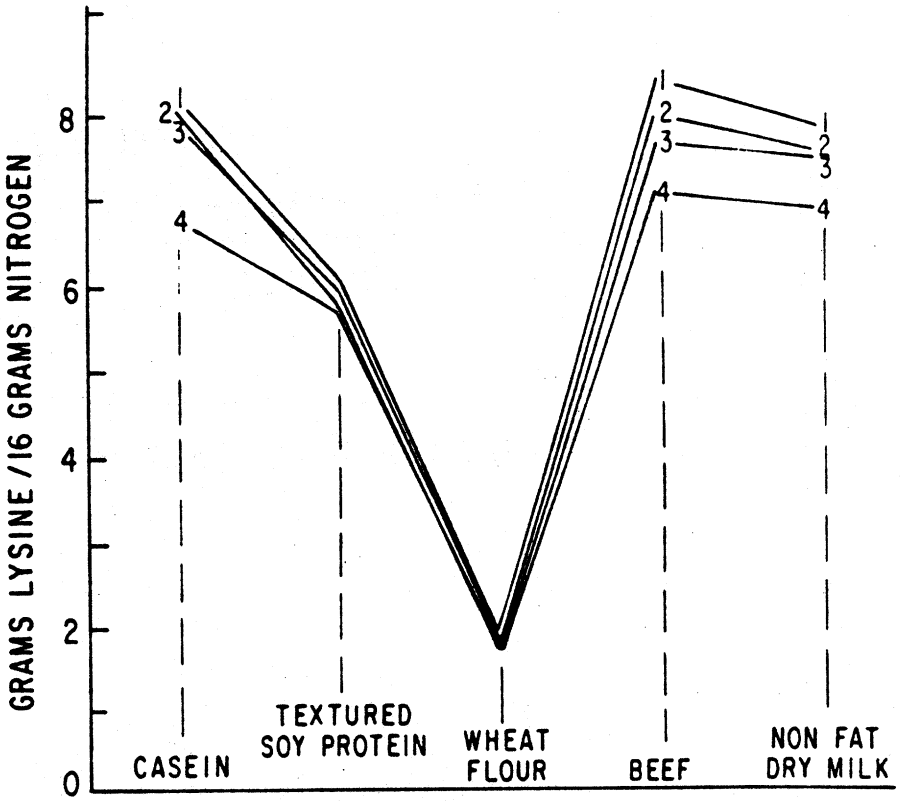

FIG. 12.3. LABORATORY-BY-PROTEIN SOURCE INTERACTIONS FOR LYSINE; AVERAGE VALUES OVER DUPLICATE HYDROLYSES AND DUPLICATE DETERMINATIONS OF EACH HYDROLYSIS (4 VALUES/POINT) FOR FIVE PROTEIN SOURCES. NUMBERED LINES REPRESENT THE FOUR LABORATORIES

protein source-by-hydrolysis interaction was due to the relatively large mean difference for lysine between hydrolyses for casein (Fig. 12.4).

The interlaboratory standard deviation for lysine over the five protein sources is 0.66, with a C.V. of 10.6%. The intralaboratory S.D. over the five protein sources was 0.13, and the C.V. was 2.1.

Inter- and intralaboratory variation data for valine (Table 12.10) is high for all five protein sources. There was one laboratory which had consistently low results on four of the sources. When those results were treated as outliers (outliers by the method of Anscombe and Tukey, 1963), the interlaboratory variation was lowered by about 50%. In all cases, the intralaboratory variation was low, indicating high precision. The precision among these four laboratories is higher for the test samples than it was among the eight laboratories for the pretest samples.

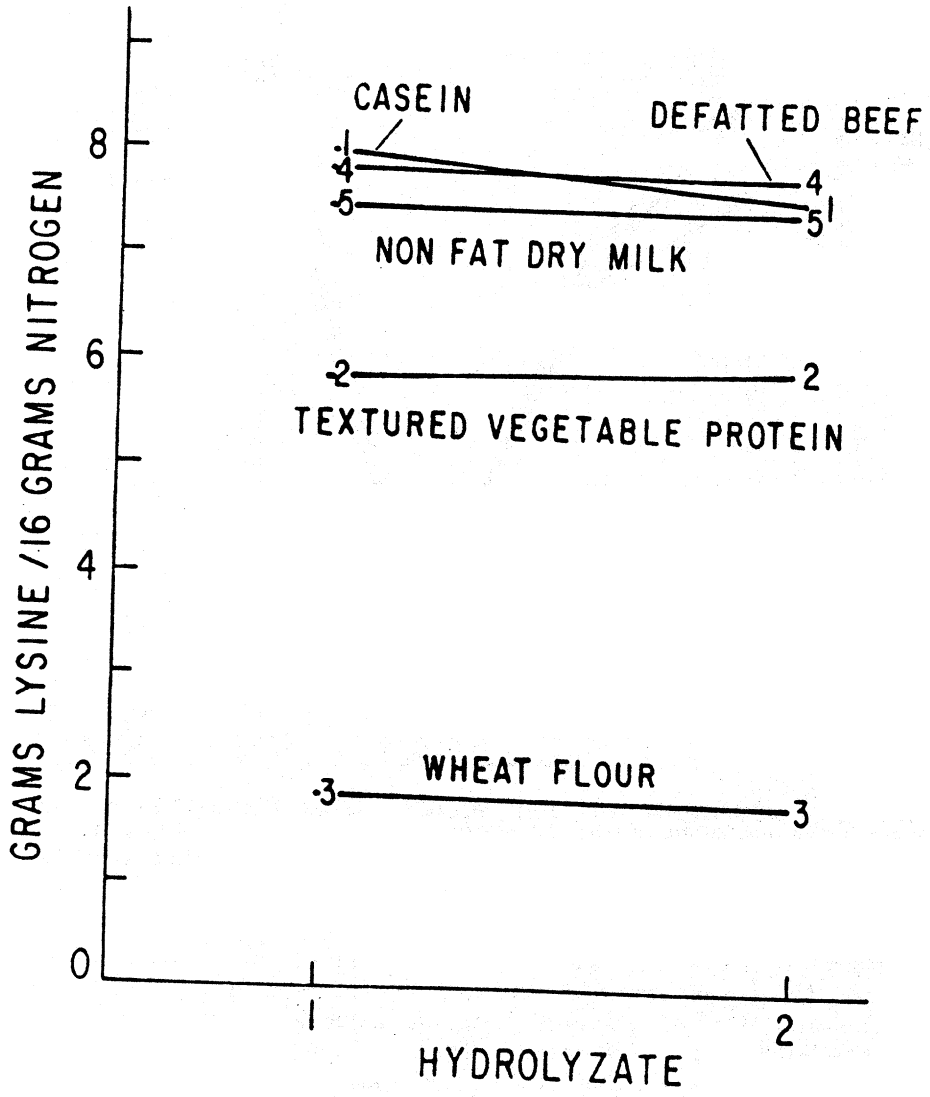

FIG. 12.4. PROTEIN SOURCE-BY-HYDROLYSIS INTERACTIONS FOR LYSINE: AVERAGED OVER DUPLICATE HYDROLYSES AND FOUR LABORATORIES (8 VALUES/POINT) FOR FIVE PROTEIN SOURCES

TABLE 12.10. INTER- AND INTRALABORATORY VARIATION FOR VALINE DETERMINATIONS OF FIVE TEST PROTEIN SOURCES[1,2]

	Casein		Textured soy protein		Wheat flour	Defatted beef		Non fat dried milk powder	
	4 Labs	Outlier removed	4 Labs	Outlier removed	4 Labs	4 Labs	Outlier removed	4 Labs	Outlier removed
Range, g AA[3]/16g N	4.26–6.80	5.20–6.80	3.00–4.33	3.68–4.33	2.90–3.60	3.37–4.70	3.80–4.70	4.28–5.80	5.06–5.80
Mean, g AA/16g N	5.75	6.13	3.89	4.06	3.28	4.18	4.42	5.16	5.50
S.D., g AA/16g N (inter-)	0.82	0.47	0.40	0.21	0.28	0.50	0.30	0.61	0.25
C.V., % (inter-)	14.42	7.66	10.21	5.19	8.54	11.92	6.71	11.84	4.52
S.D., g AA/16g N (intra-)	0.13	0.15	0.08	0.09	0.04	0.08	0.09	.09	0.11
C.V., % (intra-)	2.26	2.45	2.06	2.22	1.22	1.91	2.04	1.74	2.00

[1]Data have not been normalized (see text).
[2]Data from four laboratories, except where outlier from one laboratory was removed. Outlier data are from three laboratories.
[3]AA = amino acid.

SUMMARY

A new collaborative study on amino acid analysis was initiated with three objectives: to standardize the preparation of protein food sources for amino acid analysis, including the hydrolysis of proteins; to compare two different approaches for normalizing amino acid analysis data; and to determine the inter- and intralaboratory variation for the analysis of individual amino acids in selected protein food sources. Nine laboratories participated in the study. Seven protein sources, two as pretest samples (peanut flour and lyophilized tuna fish) and five as test samples (ANRC casein, textured soy protein, bakers' commercial wheat flour for bread, lyophilized defatted beef, and non fat dried milk powder), were prepared and analyzed. Three procedures were used for hydrolysis of the protein: 6 N hydrochloric acid for analysis of 17 common amino acids, hydroxylysine and hydroxyproline; performic acid pretreatment followed by 6 N hydrochloric acid for analysis of cystine and methionine as cysteic acid and methionine sulfone, respectively; 4.2 N sodium hydroxide for the analysis of tryptophan by the method of Hugli and Moore. Preliminary data on the two pretest samples from eight laboratories indicate the interlaboratory variation for lysine to be higher than desirable, with coefficients of variation (C.V.) of 11.3% for tuna and 11.1% for peanut flour; however, these are 6.3% and 7.6%, respectively, when one outlier is removed from the data for each of the pretest samples. The intralaboratory C.V.'s for lysine were 2.5% for tuna and 2.4% for peanut flour (8 laboratories) and 2.8% and 2.3%, respectively, with the outlier values removed. Preliminary statistical analyses of data for five test samples from four laboratories were done. These data were not normalized to nitrogen recovery or a standard amino acid profile for the reference protein casein. This will be done after data from all collaborators are received. Interlaboratory standard deviations (S.D.), expressed as grams amino acid per 16g nitrogen, for lysine were: casein, 0.65; textured soy protein, 0.25; bakers' commercial bread wheat flour, 0.09; lyophilized, defatted beef, 0.55; non fat dried milk powder, 0.36; C.V.'s were 8.4%, 4.2%, 4.9%, 7.0%, and 4.9%, respectively. The interlaboratory precision on some protein sources, therefore, was lower than desired. The intralaboratory precision was generally high with C.V.'s for lysine of: 3.0%, 4.9%, 3.8%, 3.5%, and 1.8%, respectively, for the five test samples above. The interlaboratory standard deviation for lysine determined by an analysis of variance over the five protein sources and the four laboratories was 0.66 with a C.V. of 10.6%, and the intralaboratory S.D. was 0.13 with a C.V. of 2.1%. Collaborative studies on amino acid analysis in the literature are reviewed briefly.

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DISCUSSION

DR. PELLETT: A brief question on detail. In this procedure were internal standards permitted, such as norleucine and amino β guanidino propionic acid, and if so, were they used regularly to test for ninhydrin deterioration?

MS. HAPPICH: No, we did not suggest or recommend the use of internal standards.

DR. PELLETT: Was there a special reason for that? Did you expect that the ninhydrin would stay constant or was it agreed that comparisons would be run using new reagents? In ordinary analysis, one has to take care of ninhydrin deterioration and internal standards seem to be the only way of doing this.

MS. HAPPICH: We expected that the ninhydrin would be freshly prepared and would be used soon there after. We did not expect the collaborators to use ninhydrin which was old or was deteriorated.

DR. PELLETT: I certainly think it needs to be looked at because ninhydrin doesn't only deteriorate, it often increases in its potency when freshly made, than it plateaus for a bit, and finally decreases rapidly in potency. Thus, time factors for age of ninhydrin in amino acid analysis can be important considerations.

MS. HAPPICH: They are important considerations. A calibration standard should be run, of course, each time analyses are run.

DR. STAHMANN: I want to commend Mrs. Happich and Dr. Bodwell on a very careful study which demonstrates that amino acid analysis by chromatography can be very precise and accurate. I also want to comment a bit about the tryptophan analysis. The tryptophan analysis that Moore and his group developed was based on earlier studies in which we showed you could do basic hydrolysis in cheap plastic centrifuge tubes enclosed in a glass envelope to exclude air. We also showed that tryptophan could be separated by two methods. One was to change the buffer used for elution so the tryptophan follows all other acids but this required mixing up a new buffer. A simpler way that may be somewhat easier was use of a starch column as we did; then you can use the same buffers that are used in regular amino acid analysis. Tryptophan then follows well behind all other amino acids. This does not require making up a new buffer.

As to amino acid analysis, and the question of using internal standards, this may depend upon how many standards you run. If you run a standard before and after every analysis, then you may not need an internal standard. It also may depend on how carefully the ninhydrin solution was made and particularly upon how much peroxide is present in the methyl cellosolve in which the ninhydrin is dissolved. Good methyl cellosolve from a

drum which was filled directly from the line in which it was produced gives fairly stable solutions. Old methyl cellosolve with peroxides in it give very unstable ninhydrin solutions. Thus, the need for internal standards may depend upon how the ninhydrin solution is made up.

MS. HAPPICH: That's true. Thank you, Dr. Stahmann. We in our laboratory use DMSO (dimethylsulfoxide), not methyl cellosolve, to make up the ninhydrin solution. I think that among our collaborators there may be some variation. They're probably not all using methyl cellosolve but some of them may be.

DR. SATTERLEE: One of the things that occurs during the tryptophan analysis is that lysinoalanine is formed during sodium hydroxide hydrolysis. The various collaborators should be very careful to separate it out from underneath the tryptophan peak, or else it can cause some very high tryptophan values. The starch methodology that Dr. Stahmann refers to does not have the problem of lysinoalanine interference.

MS. HAPPICH: Yes, the ion exchange method does have that problem. We did send lysinoalanine to those collaborators who requested it. They were then able to make sure that it was separated on their particular column. We run this on a short column as a very short procedure with the ion-exchange chromatographic analyzer and we do know that lysinoalanine is separated from tryptophan. Tryptophan comes off first and lysinoalanine follows, well separated. We gave the collaborators the benefit of our experience by telling them just how we separated tryptophan and lysinoalanine.